Chapter 5:

Summary and Perspectives

Current molecular definition of the neural plate border progenitor population

The neural crest has fascinated developmental biologists ever since it was first described by His in 1868 as *zwischenstrang*, or a strip of tissue between the neural tube and epidermis (Le Douarin and Kalcheim, 1999). Although inductive signals that contribute to the emergence of this cell population have been studied for some time, neural crest research in recent years has been aimed toward understanding the regulatory interactions between molecules that guide neural crest development, especially during its earliest stages. Despite a dearth of knowledge about direct regulatory relationships, many epistatic interactions between neural crest genes have been studied and information gathered from functional experiments has been compiled into a putative neural crest gene regulatory network (NC-GRN), which proposes that progressive acquisition of neural crest cell fate is driven by discrete groups of developmental regulators.

As such, the neural crest is induced at the border of the presumptive neural plate by a combination of diffusible growth factor signals that segregate neural plate from adjacent non-neural ectoderm. As a result, a group of transcription factors are activated at the junction between the two tissues that specify this region as the neural plate border, a relatively wide domain containing a heterogeneous population of progenitors with overlapping neural, neural crest, placode, and ectodermal fates. Specification of these cell fates occurs extremely early in development, even before gastrulation or bona fide neural induction. It is therefore likely that the neural plate border contains an intermixed population of multipotent stem-like cells as well as progenitors with restricted potential for the formation of specific lineages. However, it is unknown how they segregate. It is difficult to visually identify neural crest precursors

within this region because there are no known neural crest specific genes, and even canonical neural crest specifiers such as *Snail2* are expressed at pre-migratory neural crest stages by both neural crest and dorsal neural tube progenitors. In addition, expression domains of neural plate border, neural plate, ectoderm, and placodal specifiers are large and are characterized by a "salt-and-pepper" distribution. Therefore, I sought to thoroughly characterize early expression patterns of neural crest network genes in the hopes of finding an overlap between them, which may illuminate the locations of specific cell populations within this region.

I examined expression of ten members of the NC-GRN in the chick embryo during early development and found that neural crest specifiers *c-myc*, *N-myc*, *FoxD3*, and *AP-2* are expressed in the gastrula at the neural plate border. Early expression of most neural crest specifiers has not been examined previously in the chick in the context of neural crest development, and these data confirm the early specification status of this cell population. In addition, this work demonstrates conservation in timing of neural crest developmental events because studies in *Xenopus* and lamprey have also demonstrated concomitant expression of neural plate border and neural crest specifiers during gastrulation. Based on side-by-side comparison of individual gene expression domains and double *in situ* hybridization data, I propose that the chick neural plate border can be divided into posterior and anterior domains characterized by the combinatorial expression of a number of neural plate border and neural crest specifiers, which is summarized in Fig. 5.1. The anterior border of the neural plate which contains forebrain and placodal progenitors is defined by co-expression of *Zic1*, *N-myc*, *FoxD3*, *Dlx5*, *Dlx3*, and *AP-2*, which overlap with

placodal specifiers such as *Irx1*. The posterior portion of the neural plate border containing dorsal neural tube progenitors is characterized by co-expression of *Msx1*, *Pax7*, *c-myc, N-myc*, *Zic1*, *Dlx3*, and *AP-2*. As mentioned previously, the expression domains of these transcription factors are relatively large, and many of them overlap in other regions of the embryo such as the neural plate (*Zic1*, *N-myc*, *FoxD3*, *Dlx3*) and non-neural ectoderm (*Msx1*, *N-myc*, *Dlx3*, *Dlx5*, *AP-2*), suggesting potential roles in specification of several distinct cell types.

Interestingly, I noted that as development proceeds, several specifiers (*FoxD3*, *c-myc*, *AP-2*) are extinguished in neural plate border progenitors and are instead recruited to other embryonic structures, subsequently re-appearing in dorsal neural folds during late neurulation. Conversely, other genes were expressed continuously in progenitors of the dorsal neural tube but turned off in migrating neural crest cells (*Zic1*, *N-myc*). I propose that many members of the NC-GRN play separable roles in early specification and later maintenance of neural crest fate, and that activation of neural crest specifiers occurs extremely early in chick development, either concomitant with neural plate border specifiers or shortly thereafter during gastrulation. This is supported by striking similarities in expression patterns of some neural plate border and neural crest specifiers at this stage (*Msx1* and *c-myc*; *Zic1* and *FoxD3*). However, the precise timing of neural plate border and neural crest specifier induction is still unknown, and resolving this question necessitates further *in situ* hybridization and QPCR analysis of gene expression at developmental stages preceding HH4.

Prospects for cellular resolution at the neural plate border

While these studies bring us closer to defining the neural plate border region, we are still unable to resolve gene expression on a single-cell basis. Therefore, we are currently optimizing fluorescent *in situ* hybridization techniques that will enable us to obtain cellular resolution of combinatorial expression of up to three specifier genes at once (Denkers et al., 2004). In such an experiment, it will be important to define the overlap between markers of neural plate, non-neural ectoderm, neural plate border, and placodes with high resolution which is not possible by chromogenic means. Ultimately, the best approach to this question would involve live imaging of chick embryos expressing fluorescent reporter constructs driven by specific neural crest gene enhancers, enabling precise cellular resolution of spatiotemporal changes in gene expression as they occur during development. Such neural crest gene specific enhancer elements are currently being investigated in our laboratory, and the comprehensive characterization of spatiotemporal expression patterns of chick neural crest genes conducted here will serve as valuable background for further identification of potential regulatory elements.

Discovery of epigenetic regulators in the developing chick embryo

In light of these findings, it is intriguing that despite exposure to a plethora of regulatory signals from the time of specification to terminal differentiation several days later, neural crest cells maintain some degree of multipotency, even upon reaching their targets. The multipotent progenitor state of the neural crest has been demonstrated by a number of lineage tracing, back-transplantation, and clonogenic studies; however, potential mechanisms

responsible for maintenance of this plastic state have been elusive (Crane and Trainor, 2006). Therefore, we hypothesized that members of the NC-GRN may be regulated by a yet-unknown mechanism that serves to prevent premature lineage decisions and differentiation. The Polycomb group (PcG) of epigenetic repressors emerged as promising candidates because these proteins have been demonstrated to mediate global repression of developmental regulator genes in a variety of stem cells and stem-like progenitors, including mouse and human embryonic stem cells, fibroblasts, hematopoietic stem cells, and cancers (Sparmann and van Lohuizen, 2006). In addition, the PcG has been shown to be necessary during lineage restriction and differentiation by repressing pluripotency factors and regulators of alternative cell fates. Although epigenetic repression mechanisms have not been investigated during *in vivo* neural crest development, neural crest derived neuroblastomas often exhibit dysregulation of chromatin-modifying genes, including Polycomb, consistent with possible involvement in normal development (Cui et al., 2006).

Therefore, I first set out to examine whether PcG genes are expressed at the right time and place for participation in neural crest development. I found that Polycomb Repressive Complex 2 (PRC2) members *Suz12* and *Eed* and Polycomb Repressive Complex 1 (PRC1) proteins *Bmi-1*, *Ring1B*, *Cbx2*, *Cbx8*, and *Phc1* are expressed throughout early chick development in large domains that include the neural plate border, neural folds, and migrating neural crest, which is summarized in Fig. 5.1. While some of the PRC gene expression patterns were more specific than others, none were ubiquitously expressed at all stages. Widespread PcG expression in the gastrula is strongly suggestive of a role in maintenance of multipotency and regulation of tissue specification signals.

Interestingly, I found that all seven Polycomb genes examined are localized at high levels in the anterior neural folds, which do not express neural crest specifiers and do not generate neural crest. We hypothesize that the PcG, in combination with anteriorizing neural signals such as Wnt inhibitors, may act to repress neural crest cell fate in anterior neural folds, possibly by selectively inhibiting neural crest regulator genes while leaving forebrain and placode specifiers unaffected. Furthermore, specific PcG expression in migratory neural crest cells, which are characterized by rapid proliferation and a high degree of cell fate plasticity, suggests that Polycomb-mediated epigenetic repression may be involved in regulating these processes as well. Therefore, the PcG might function to modulate a number of processes during neural crest development, likely by negatively regulating NC-GRN genes.

In vivo functional analysis of Polycomb Group factor Bmi-1

To investigate the *in vivo* function of Polycomb proteins in the chick neural crest, we undertook a morpholino (MO) knock-down approach focusing on the PRC1 member Bmi-1, which has been shown to regulate self-renewal of neural, hematopoietic, and enteric neural crest cells in culture (Molofsky et al., 2003). We found that Bmi-1 MO electroporation into the prospective neural plate border during gastrulation results in consistent upregulation of *Msx1*, *FoxD3*, and *Sox9* transcripts by early neurulation stage HH6. In the case of *Msx1*, which showed an obvious phenotype by *in situ* hybridization, the Bmi-1 MO-induced increase in expression is not due to ectopic expansion of the *Msx1* domain or an increase in cell proliferation, but is a direct result of an increase in expression within the endogenous neural fold territory. The visible enhancement of *Msx1*

expression is likely a consequence of either an increase in intracellular transcript concentrations or an increase in the number of neural plate border and neural fold cells that are recruited to express it. Due to the lack of cellular resolution of gene expression and the heterogeneity of the target cell population, we cannot yet distinguish between these alternate possibilities, which are not mutually exclusive. However, we can definitively conclude that Bmi-1 represses *Msx1* because its expression is conversely decreased in embryos overexpressing high levels of Bmi-1 protein and its partner Ring1B.

Likewise, Bmi-1 MO knock-down results in an increase in *FoxD3* and *Sox9* transcript quantities at early neurulation stage HH6 as measured by QPCR. This derepression leads to *FoxD3* and *Sox9* being expressed during HH6 at levels which are not usually seen until much later in development, and may cause premature commitment or segregation to the dorsal neural tube/neural crest lineage. However, we have been unable to examine how these transcriptional changes functionally affect neural crest development because early transcriptional derepression leads to misregulation of neural crest specifiers at later stages. This secondary effect probably results from a perturbation of the delicate balance between these factors, which is controlled by highly complex cross- and auto-regulatory interactions. In addition, by the time that neural crest cells begin migrating in electroporated embryos, this highly plastic cell population has compensated for the early phenotype, such that no effect on migration or differentiation is observable.

Therefore, we plan to continue to examine the role of Bmi-1 in neural crest differentiation and proliferation in an *in vitro* explant culture system, which will allow us to minimize the number of variables confounding this question. A

homogeneous population of isolated neural crest stem-like cells, such as EPI-NCSC, would present an ideal system (Sieber-Blum and Hu, 2008). However, it may be also possible to use a culture system such as demonstrated by Basch et al., in which explants of tissue containing neural crest progenitors are cultured for a week in the absence of exogenous factors until differentiation occurs autonomously. We have attempted to replicate this system and have found that explants of gastrula-stage medial epiblast and neurula-stage midbrain dorsal neural folds generate migratory neural crest cells that express the HNK-1 antigen within 36 hours in growth factor restricted culture medium. Although we were unable to observe a difference in neural crest cell emigration from explants that have been electroporated with Bmi-1 MO as compared to control MO (data not shown), we also have not examined these cultures for changes in expression of differentiation markers, and plan to repeat these experiments using a more stringent knock-down approach. For example, we will attempt to inactivate the upstream PRC2 complex in neural crest cell cultures with the chemical inhibitor DZNep, which has been shown to effectively and specifically disrupt PRC2 function in cancer cells, and examine the effect on cell survival, proliferation, and differentiation (Tan et al., 2007).

Optimizing Polycomb loss-of-function approaches

We are significantly limited by the fact that our perturbations are specific to one member of the downstream complex of a large bipartite protein group, and although some biochemical studies have shown that knock-down of single PRC members can disrupt activity of the entire complex, PRC1 proteins may function somewhat redundantly during development (Lee et al., 2006).

Therefore, in future knock-down experiments we will apply several PRC1 morpholinos together, targeting Bmi-1 along with the catalytically active partner Ring1B and the specifically expressed PRC1 member Phc1. Co-electroporation of three morpholinos at high concentrations in the gastrula has been shown to result in viable chick embryos with a drastic and specific neural crest phenotype at later stages (Betancur and Sauka-Spengler, personal communication). Experiments with three PRC1 morpholinos will undoubtedly result in a stronger phenotype and may lead to more obvious effects on later developmental events.

In addition, recent development of shRNA cassettes driven by discrete neural crest-specific enhancers in our laboratory shows great promise for studies in which target genes necessitate inactivation in a cell-specific, temporally controlled manner. These neural crest-specific enhancer elements drive mir-shRNA constructs at levels comparable to endogenous expression of the targeted gene, which overcomes the problem of non-specific effects that have been reported with ubiquitously driven shRNA by us and others (data not shown, Mende et al., 2008). This approach may allow us to examine late effects of Bmi-1 knock-down and to overcome early specificity and compensation issues.

Strategies for large-scale analysis of Polycomb function

In the meantime, it will be interesting to examine whether PRC1 knock-down also affects expression of other specifier genes in the chick embryo in a similar manner to *Msx1*, *FoxD3*, and *Sox9*. For instance, are other neural plate border specifiers affected? We did not observe a significant effect of Bmi-1 MO on expression of *Pax7*, which may suggest that either *Msx1* is repressed specifically, or that *Pax7* is less sensitive to the effects of Bmi-1, which we may be

able to resolve in a double or triple PRC1 knock-down experiment. Does the Polycomb complex repress neural crest network genes that have been shown to stimulate proliferation and inhibit differentiation, such as *Zic1*, *c-myc*, *AP-2*, and *Id2*? It is possible that these genes cooperate with PRC1 during stages of neural crest development that necessitate extensive proliferation and multipotency (i.e., migration), and are therefore not repressed at that time. Are specifiers of other neural plate border fates, such as placodes, also regulated? We presume that placodal specifiers such as *Irx1* would be repressed by PRC1, given that Bmi-1 and its partners are co-expressed with these genes in the pre-placodal region. Does Polycomb knock-down affect the cell cycle of neural crest progenitors in a way that cannot be detected by phospho-histone H3 immunostaining? Are factors that regulate AP patterning in the chick embryo, such as Hox genes, Krox20, and Wnt, dysregulated in these experiments?

In order to examine whether PRC1 knock-down affects NC-GRN genes specifically or globally, we plan to assay electroporated embryos at HH6 for changes in expression of a number of other neural plate border and neural crest specifiers, induction factors, ectoderm, placode, neural plate, and axial patterning specifiers, and mitotic and apoptotic markers. To this end, we will use the NanoString nCounter Gene Expression Assay system, which allows for large-scale multiplex quantitative analysis of mRNA expression directly without the necessity for RNA extraction, reverse transcription or amplification procedures (Geiss et al., 2008; Su et al., 2009). This incredibly sensitive assay can also be used to quantify changes in gene expression as a result of PRC1 over-expression, either alone or in combination with Bmi-1 MO in a rescue experiment. We imagine that Bmi-1 and other PRC1 partners repress developmental regulator

genes in the chick embryo selectively, due to the presence of other complex regulatory interactions, unlike the global repression which has been observed in homogeneous ESC populations.

However, definitive conclusions about direct regulation of NC-GRN genes by PRC1 will require experimental evidence of association of Polycomb proteins with the chromatin context of target genes and simultaneous presence of repressive histone methylation marks. Therefore, we plan to first investigate whether PRC1 members such as Bmi-1, Ring1B, Cbx8, and the PRC2 member Suz12 are associated with upstream regulatory regions of Msx1, FoxD3, and Sox9 during early chick development by chromatin immunoprecipitation (ChIP). This experiment will allow us to determine whether the neural crest specifiers are repressed by Bmi-1 directly or secondarily through Msx1 regulation. We have identified antibodies that immunoprecipitate PRC1 complex partners from protein extracts of chick embryos, and are currently optimizing chromatin sonication conditions and cross-linking procedures in order to minimize background and increase specific signal. We are also testing specific primer sets that we have designed within highly conserved upstream regulatory sequences of Msx1 and FoxD3 that have shown PcG occupancy in human and mouse ESC ChIP assays (Stock et al., 2007). Additionally, we would like to address whether de-repression of neural crest genes observed in Bmi-1 knock-down experiments occurs as a consequence of the removal of Polycomb complexes and H3K27me^3 marks from chromatin. Finally, an ideal experiment would involve large-scale investigation of PcG chromatin association during several distinct stages of chick development using the ChIP-on-Chip method, allowing us to determine how epigenetic regulation of lineage specifier genes changes in the embryo with time.

Alternative splicing as an additional regulatory mechanism

In addition to the challenges of studying a large, multifunctional complex of epigenetic regulator proteins *in vivo*, we discovered another level of complexity when we isolated several truncated splice isoforms of Bmi-1 from a chick cDNA library. Understanding the splicing events responsible for generating these variants necessitated thorough characterization of the chick Bmi-1 genomic region, which lies on an unassigned chromosome and is not annotated in the chick genome. We have found that truncated variant V4 contains the conserved RING finger domain which is necessary for interaction between Bmi-1 and Ring1B proteins, but lacks a nuclear localization signal (NLS) and other C-terminal functional domains. In contrast, variant V6 lacks the N-terminal RING finger but contains a NLS and the helix-turn-helix-turn-helix-turn (HTHTHT) motif which mediates interaction with the Ph proteins and is responsible for repressive activity; however, a small portions of the HTHTHT motif is missing in V6, suggesting possible reduced functionality.

Preliminary expression studies using QPCR have demonstrated that *V6* is expressed at similar levels as full-length *Bmi-1* during early development, suggesting that it may be a positive regulator of its expression. In addition, we have found that *V4* transcripts are present throughout early chick development at levels significantly below those of full-length Bmi-1, and that over-expression of this variant at the gastrula stage recapitulates the effect of Bmi-1 MO on *Msx1* expression. We predict that it functions in this manner by binding to full-length Bmi-1 and the Ring proteins and preventing them from engaging in the PRC1 complex and translocating to the nucleus. It would be interesting to investigate whether V4 inhibits Bmi-1 activity in a cell type-specific manner, or whether it

acts more generally within the neural fold to restrict Bmi-1 activity to a critical level.

Unfortunately, the RT-PCR techniques used in our experiments do not provide spatial information about variant expression, and we are unable at present to distinguish whether the Bmi-1 isoforms function specifically within the neural crest progenitor population or are differentially expressed in other tissues. Specific variant probe synthesis for *in situ* hybridization proves problematic due to the small size of truncated isoforms and large regions of homology shared between them, especially within 3'-UTR. In addition, variant transcripts may be present at levels below detection by standard whole-mount *in situ* hybridization technique. Therefore, we have designed fluorescently labeled locked nucleic acid (LNA) probes, which are highly stable RNA analogs with high affinities toward even very short complementary sequences, and which have been used successfully to analyze microRNA expression in vertebrate embryos by whole-mount *in situ* hybridization (Kloosterman et al., 2006; Kubota et al., 2006). We are currently testing these LNA probes in the chick embryo using an *in situ* hybridization technique that includes additional signal amplification steps.

Although we have not yet obtained spatiotemporal resolution of V4 expression or tested its ability to interact with other PRC1 proteins, the data generated so far strongly suggest that V4 may be a naturally occurring dominant-negative modulator of Bmi-1 activity. We next plan to examine the biochemical mechanism by which V4 functions in a cell culture system using bimolecular fluorescence complementation analysis (BiFC), which has been successful in visualizing protein interactions in live cells (Hu and Kerppola, 2003;

Grinberg et al., 2004; Shyu et al., 2006). We are currently making fluorescent fusion constructs containing full-length Bmi-1, truncated variants, and other PRC1 members such as Ring1B and Phc1 together with truncated fluorophores, which will be transfected into live cells for imaging studies. We expect that V4 associates with full-length Bmi-1 and Ring1B via the RING finger domain, but is not able to interact with Phc1 or to translocate to the nucleus. In contrast, V6 should not be able to interact with Ring1B, but may bind Phc1. However, since the V6 translation initiation site is located within the HTHTHT domain, the translated V6 protein lacks five amino acids of this protein interaction motif, and it will be interesting to examine whether its affinity for interaction with Phc1 is reduced. If the truncated HTHTHT domain of V6 retains protein-binding and repressive activity, it is possible that V6 may act as a substitute for Bmi-1 in PRC1 complexes. Alternatively, V6 may be able to bind to Bmi-1 and stimulate its activity within the complex. In addition, the culture experiments should be able to demonstrate whether the alternative putative NLS located near the C-terminus of V6 is functional, giving this variant the ability to translocate to the nucleus. Luciferase assays may be useful for determining whether the HTHTHT domain truncation reduces the repressive activity of V6.

Finally, we also plan to investigate the function of V6 *in vivo* by over-expression. We have prepared a pCIG-V6-GFP construct, which we plan to inject into HH4 embryos for analysis at HH6 and HH10, stages when endogenous levels of V6 are relatively low, as demonstrated by QPCR. Alternatively, we may overexpress V6 at HH6 and assay electroporated embryos for changes in neural crest gene expression at later stages. Based on the similarity in expression levels of V6 and full-length Bmi-1, as well as the presence of a putative NLS and the

HTHTHT repressive domain in this variant, we predict that over-expression would result in a gain-of-function phenotype similar to that observed with Bmi-1 and Ring1B co-electroporation. If this variant indeed functions in a dominant-active manner, it is possible that it may be compensating for full-length Bmi-1 in our MO knock-down experiments, explaining the weak phenotype that we observe. In addition, the high expression peak of V6 at HH8 may explain why we do not see a consistent effect on neural crest network genes when we analyze Bmi-1 MO-electroporated embryos at later stages. Therefore, we also plan to design a morpholino targeting the unique V6 start site for use in co-electroporation experiments with the 5' Bmi-1 MO (as well as with MOs against other PRC1 members).

Although these studies are far from complete, they present novel evidence for regulation of Bmi-1 by alternative splicing. Given the high incidence of strongly conserved splice variants within large protein families involved in key aspects of development, such as FGF, Dlx, and Pax, it is likely that modulation of protein function by alternative splicing is a common regulatory mechanism in vertebrate development. Accordingly, inappropriate expression of splice isoforms of PcG members has been demonstrated in cancers, highlighting the importance of precise control of Polycomb activity and critical involvement of alternatively spliced variants in developmental processes.

Conclusions

In summary, in my thesis project I have demonstrated that a number of neural crest specifiers are co-expressed in the neural plate border of the chick gastrula with early neural plate border specifier genes, which has contributed to

the identification of this region by combinatorial gene expression. I have also investigated how neural crest network gene expression domains resolve over time and found that most specification signals are continuously present throughout neural crest development, suggesting a need for upstream regulation. I have demonstrated that members of the Polycomb Group of epigenetic regulators are co-expressed with neural crest network genes throughout early chick development, and that the Polycomb Repressive Complex 1 member Bmi-1 functions to negatively regulate *Msx1*, *FoxD3*, and *Sox9* in the neural plate border of the chick neurula. Finally, I have characterized the Bmi-1 genomic locus and identified several truncated splice variants which are expressed during early development, and have demonstrated that one of the variants possesses dominant-negative activity *in vivo*. Therefore, I have characterized some of the molecular and epigenetic events that participate in neural crest formation and have found that multiple levels of regulation, including genetic, epigenetic, and biochemical inputs, are involved in development of this cell population.

Figure 5.1: NC-GRN and PcG expression during early development

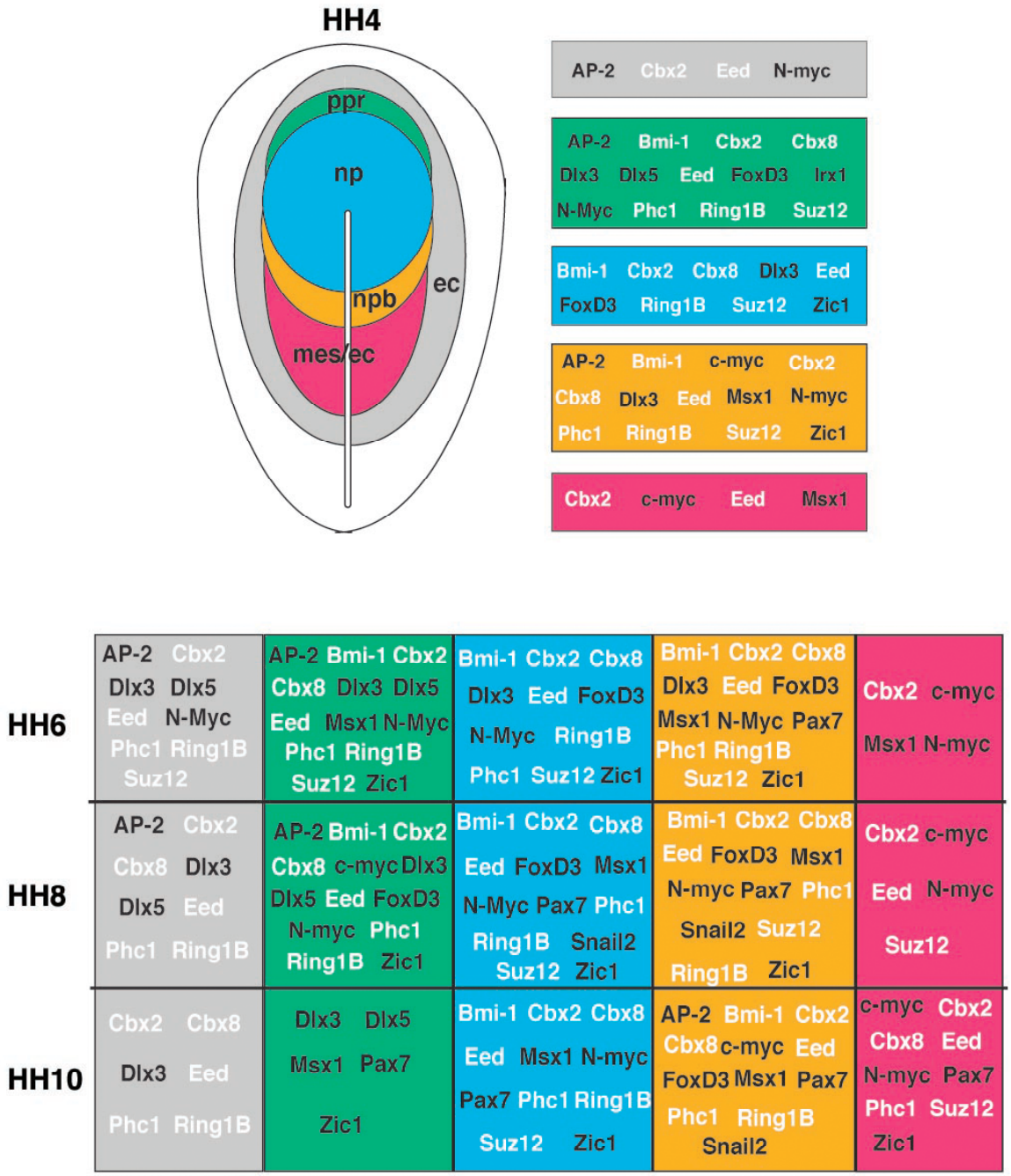

Fig. 5.1. Schematic diagram illustrating respective locations of the following presumptive tissues in the chick gastrula: non-neural ectoderm (gray), pre-placodal and anterior neural region (green), neural plate (blue), neural plate

border (orange), and posterior mesectoderm (pink). Neural crest network genes (black type) and Polycomb Group genes (white type), which were found to be expressed in each of these subregions, are listed in color-coded blocks. A summary color-coded chart lists the neural crest and Polycomb genes which were found to be expressed in progenitors of the aforementioned lineages at HH6, HH8, and HH10. Note that many genes share expression in closely apposed tissues, such as neural (dorsal neural tube) and neural plate border (neural crest).